Finally, a word of caution concerning interpretation of isoelectric focusing patterns is in order. It is conceivable that a given protein may interact with the supporting ampholyte to give multiple banding. Also, the considerations presented above for zone electrophoresis are equally applicable to chromatography. Possible complications of this sort will become apparent, however, when the fractions themselves are analyzed to see whether they run true.

[12] The Applicability of Acrylamide Gel Electrophoresis to Determination of Protein Purity

By Robert F. Peterson

Acrylamide gel electrophoresis has been covered extensively by several authors in this series of volumes in articles on analytical disc electrophoresis, slab vertical cells, and preparative disc electrophoresis.[1-4] At this time it would seem appropriate to summarize the experience of the past several years and detail the precautions to be taken with this widely used procedure. The two basic questions we ask of analytical gel electrophoresis are: Is a protein pure if it migrates as a single band? and: Is it necessarily heterogeneous if it shows more than one band? At each step of the procedures we shall discuss the precautions necessary to avoid misleading results.

All the chemicals used for acrylamide gel electrophoresis, excepting buffer components, should be stored at refrigerator temperatures, 0–5° to prevent deterioration. Acrylamide monomer and the cross-linking agent, methylenebisacrylamide, as well as the commercial mixture of these, Cyanogum-41,[5] slowly polymerize, resulting in decreased solubility in buffer solutions. Ammonium persulfate should be tested by measuring the pH of a 1% solution in water; if the pH is below 2, a new batch should be used. The amines which are used for the other half of the catalyst pair discolor with time. If a catalyst, such as 0.2% ammonium persulfate and 0.1% *N,N,N′,N′*-tetramethylethylenediamine (TEMED), is not active enough to gel 5% acrylamide solution within 20 minutes, the gel produced in a slab cell may be heterogeneous, resulting in uneven mobilities across its width for identical samples.

The high resolution of gel electrophoresis methods is due to the production of very thin starting zones, followed by imposition of the constraint of molecular sieving on movement due to the electrical force field. The species to be examined must enter the gel. If an examination of the dyed gel shows a heavy residue where the sample should have entered the gel, the pore size is too small. Blattler and Reithel[6] have published a table correlating molecular weights of proteins which are totally excluded by gels to the acrylamide concentration. A series of experiments with decreasing concentrations of acrylamide will provide data for choosing a gel which will give the optimum separation of components.

When the proteins are highly aggregated, urea and often 2-mercaptoethanol are used to obtain monomers of the proteins. If analytical grade urea, low in cyanate, is used and the buffer solutions are acidic, carbamylation of the lysine groups of the protein will not occur. However, at alkaline pH there is always a possibility that artifactual bands may be produced. Urea solutions may be passed through mixed-bed ion exchangers (Amberlite MB-1 with 5% IR-128 added) to remove cyanate.[7] A protein solution, once dissolved in urea at alkaline pH, should be used immediately. If carbamylation is suspected, cyanate may be added to protein samples to see whether the suspected bands are intensified. Guanidine hydrochloride cannot be used as a dissociating agent because of the high conductivity of its solutions. Sodium dodecyl sulfate (SDS) is an excellent dispersing agent, but completely alters the determination, since small charge differences are equalized owing to the high adsorption of SDS.

Electrophoresis in buffers containing 0.1% SDS and mercaptoethanol at pH 7–9 is an excellent method for determining monomer chain molecular weights[8] (cf. Weber, Part C). Because of the high adsorption of SDS, averaging 1.4 g per gram of protein, differences in net charge due to amino acid replacement are imperceptible, and mobility is strictly a function of molecular weight. The logarithm of molecular weight plotted against distance moved in the gel is a straight-line function. Heterogeneity, due to higher or lower molecular weight contaminants, is thus easily detected.

The choice of buffers may be illustrated with protein polymorphs which differ by replacement of a single charged amino acid. The series of β-caseins A^1, A^2, and A^3, which contain respectively 6, 5, and 4 histidine residues per molecule of 24,000 molecular weight, can be resolved in 10% Cyanogum-41 gels at pH 3 in formic–acetic acid buffers containing 4.5 M urea. The same β-caseins are not resolved at pH 9 because the histidine side chains are not charged. Analysis at a single pH is not a sufficient test for heterogeneity.[9]

Oxygen inhibits polymerization. For this reason deaerated water is used to cover the acrylamide solutions being polymerized in tubes. In flat slab cells, such as those of Raymond's design,[10] air is excluded by rubber gaskets. The area exposed around the slot formers will not gel completely unless the assembled cell is put in a polyethylene bag and nitrogen is used to flush out the air. When acrylamide concentrations are materially below 5%, this precaution may be essential.

Catalyzing the polymerization of acid solutions of acrylamide can be accomplished by increasing the proportion of amine to persulfate over the amount used in alkaline solutions. However, the amine increases the pH of the buffer and necessitates a lengthy prerun to remove the catalyst by-products from the gel. In this laboratory we use 0.5 ml of 30% hydrogen peroxide and 0.35 g of thiourea per 100 ml of acrylamide solution. The hydrogen peroxide cannot be detected with tolidine–KI reagent a few minutes after mixing and makes no contribution to the ionic strength of the buffers. Therefore, the necessity of lengthy preruns to reduce the conductivity of the gels is eliminated.

In water-cooled cells higher voltages may be applied, with consequent improvement in resolving power. If identical samples produce a curved front across a gel slab, the voltage is too high. In analytical gels the proteins are usually rendered visible by dyeing the gel in acetic acid solutions of an acid dye. If the protein is not precipitated by acetic acid or is soluble in acetic acid, this procedure will not work. Amido Black 10B (Color Index 20470) has been used by many workers. Insufficient dyeing time will cause a concentrated protein band to appear as two bands. A recent method of Fazekas de St. Groth consists of fixing and dyeing simultaneously in 0.025% solution of Coomassie Brilliant Blue which contains 10% trichloroacetic acid in acetic acid–methanol–water (14:40:160, v/v/v).[11] The acrylamide gels are dyed for 24 hours and then washed free of excess dye with acetic acid–methanol–water (14:40:160, v/v/v). In our laboratory this is very effective for the detection of protein at low concentration and is successful in dyeing proteins complexed with SDS in molecular weight determination gels.

Until the advent of scaled-up disc electrophoresis as a preparative method, proteins had been detected by dye binding and had not been subjected to chemical analysis. Work in this laboratory showed that casein components, isolated from urea-containing gels, were being extensively degraded. A prerun removed persulfate ions from the gel. However, it did not stop the degradation; nor did photopolymerizing the acrylamide preparative columns. However, a prerun with hydroquinone would scavenge the gels and proteins could be recovered without damage.[12]

Because the termination step in free-radical polymerization involves the combination of two free radicals to form a homopolar bond (and this might not be possible in a rigidly cross-bonded gel), free radicals may exist for a long time after gel polymerization. When acrylamide monomer was polymerized in aqueous solution using the γ-ray flux from the Yale electron accelerator, a strong electron spin resonance (ESR) signal was obtained at 3400 gauss. Persulfate-amine polymerized gels had a weaker resonance at the same point, and this was not decreased by lengthy electrophoresis to remove the persulfate. When caseins were analyzed for tryptophan, this amino acid was found to be totally destroyed after preparative gel electrophoresis in 10% acrylamide gels. β-Lactoglobulin was eluted as a symmetrical peak from 10% acrylamide gels which did not contain urea. Nevertheless, the tryptophan content was considerably reduced. Histidine and tyrosine were essentially unaffected by preparative gel electrophoresis. Again, a preliminary run with a scavenging agent such as cysteine, thioglycolate, or hydroquinone would prevent damage to proteins during preparative electrophoresis on acrylamide gels.[13]

A prerun interferes with the stacking gel-running gel systems.[2] However, Hjertén and co-authors[14] pointed out in 1965 that equal resolution could be obtained in single running gel systems, if the sample of protein had a lower conductivity than the following buffer. The practice of embedding the sample in a separate gel, which is photopolymerized with a riboflavin catalyst, has been discarded in most preparative work, since addition of sucrose to the sample will produce good layering on the running gel. Pastewka and co-workers[15] have recently noted that exposure of hemoglobin solutions to the fluorescent light used for riboflavin-catalyzed polymerization of stacking gels resulted in the formation of artifacts.

Cann (cf. [11]) has shown that artifacts can be produced by protein–buffer interactions in gel electrophoresis.[16] Bovine serum albumin may show two or three zones due to reversible protein–borate buffer interactions. Conalbumin also exhibits this behavior. Myoglobin, however, has been separated by pH-gradient preparative electrophoresis into three components which may be identical with the species previously suspected to be buffer interaction artifacts.[17]

With all the possible causes mentioned for artifacts, no infallible method exists for determining heterogeneity. Analytical gel electrophoresis at acid and basic pH are a minimum for asserting purity. Analytical molecular weight determination in SDS gels is sensitive to small amounts of impurities which differ in molecular weight.

We still have no detection method for genetic variants which involve nonpolar amino acids. The mobility differences observed by Kalan *et al.*[18] for two genetic forms of swine whey protein, which differ only in alanine and valine content, can perhaps be attributed to changes in the environment of charged groups.